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PRIMER NOTE

Microsatellite markers for *Vaccinium* from EST and genomic libraries

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Abstract

We present 30 microsatellite loci isolated from expressed sequence tag (EST) and genomic libraries in *Vaccinium corymbosum* L. Allele number per locus in 11 tetraploid and one diploid *V. corymbosum* accessions ranged from two to 15 (mean = 8.16) in 24 single-locus simple sequence repeats (SSRs). Cross-species amplification in a panel of 12 species representing nine sections ranged from 30 to 100% (mean = 83%).

Keywords: blueberry, EST, microsatellite, PCR, SSR, *Vaccinium*

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Vaccinium (Ericaceae) is a widely distributed plant genus found on all continents except Australia and Antarctica and exhibits a high level of morphological diversity. Fruits from several *Vaccinium* species are collected from the wild for food. *Vaccinium* species in section (§) *Cyanococcus* are used to develop blueberry cultivars, which are grown for their edible fruit (Vander Kloet 1988). Highbush blueberry cultivars, *Vaccinium corymbosum* L., are tetraploid ($2n = 4x = 48$). They are vegetatively propagated and can be easily misidentified based on phenotype. Our objective was to develop simple sequence repeat (SSR) markers for use in identifying blueberry germplasm held at the US Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository in Corvallis, Oregon.

SSR markers were derived from two expressed sequence tag (EST) libraries and from a microsatellite-enriched genomic library, constructed from *V. corymbosum* cv. Bluecrop DNA. EST libraries were constructed from cold acclimated (CA) and nonacclimated (NA) floral buds (Rowland *et al*. 2003; Dhanaraj *et al*. 2004). For the genomic library, DNA from young leaves was extracted with the PureGene kit (Gentra) followed by phenol–chloroform extraction (Sambrook *et al*. 1989). Genomic DNA was enriched using an oligonucleotide mix of $(AGA)_{20}$, $(CTT)_{20}$, $(TG)_{20}$, $(GA)_{20}$, $(CT)_{20}$ and $(AC)_{20}$. SSR enrichment was based on a modified protocol (available at http://www.uga.edu/srel/DNA_Lab/protocols.htm) of Hamilton *et al*. (1999). In brief, DNA was digested with *Rsa*1, ligated to double stranded superSNX linkers, hybridized with biotinylated microsatellite oligonucleotides and captured on streptavidin coated magnetic beads. Unwanted DNA was washed away, and captured DNA was recovered by polymerase chain reaction (PCR) using the single stranded superSNX-f as a primer. The PCR products were ligated into the pCR®4-TOPO® vector and cloned into the TOPO TA Cloning® kit version K (Invitrogen). PCR was used to determine the insert size and DNA was purified from colonies containing 500–1000 bp inserts using the Perfectprep Plasmid 96 Spin DB kit (Eppendorf). Inserts were sequenced on a CEQ 8000 genetic analyser (Beckman-Coulter) using primers TopoF: 5′-CGCCAAG-CTCAGAATTAACCCTCAC-3′ and TopoR: 5′-CGACG-GCCAGTGAATTGTAATACG-3′.

A total of 1305 EST sequences from both CA and NA libraries and 136 SSR-enriched genomic sequences (prefix = VCC for *Vaccinium corymbosum* Corvallis) were clustered using CAP 3 software (Xiaoqiu & Madan 1999) and screened for the presence of microsatellites with five or more perfect repeats with the SSRIT software (Temnykh *et al*. 2001). The BLASTN and BLASTX algorithms (Altschul *et al*. 1997) were used to identify homologous sequences in GenBank. Primers were designed using PRIMER 3 software (Rozen & Skaletsky 2000). A 'pigtail' was added to the 5′ end of most reverse primers to ensure adenylation of the 3′ end of the forward strand by *Taq* polymerase (Brownstein *et al*. 1996).

Amplification and polymorphism of each primer pair were evaluated on 11 tetraploid *V. corymbosum* cultivars ('Grover', 'Pioneer', 'Rancocas', 'USDA-72', 'Cabot', 'Bluecrop', 'Georgiagem', 'Earliblue', 'Flordablue' and 'Toro') and one accession of wild *V. corymbosum* (PI 55880) (Table 1). A

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Table 1 Locus name, GenBank Accession no., repeat motif, primer sequences (F = forward, R = reverse), annealing temperature (T_a), allele size range and number of alleles per locus of 30 *Vaccinium* SSR loci evaluated in 11 *Vaccinium corymbosum* cultivars ('Grover', 'Pioneer', 'Rancocas', USDA-72, 'Cabot', 'Bluecrop', 'Georgiagem', 'Earliblue', 'Flordablue' and 'Toro') and one accession of wild blueberry (PI 55880). Locus name prefixes indicate source: CA, cold acclimated EST; NA, nonacclimated EST; VCC, genomic enriched; ND, not determined

Locus	GenBank accession no.	Repeat motif	Primer sequence	T_a (°C)	Allele size range (bp)	Allele no.
CA23F	CF810543	$(AGA)_6$	F: GAGAGGGTTTCGAGGAGGAG R: GTTTAGAAACGGGACTGTGAGACG	62	150–170	2
CA112F	CF810443	$(AG)_7$	F: TCCACCCACTTCACAGTTCA R: GTTTATTGGGAGGGAATTGGAAAC	62	140–200	5
CA169F	CF811071	$(GAT)_4$	F: TAGTGGAGGGTTTTGCTTGG R: GTTTATCGAAGCGAAGGTCAAAGA	62	109–130	5
CA190R	CF811085	$(TGC)_5$	F: TTATGCTTGCCATGGTGGTA R: TTGCGAAGGGACCTAGTAGC	62	250–280	3
CA236F	CF810540	$(TG)_{17}$	F: GTTAAGCTTTTAGATGAGTTGATGG R: GTTTAACCAGTCCCAGACCCAAAT	64	250–280	6
CA344F	CF810639	$(GCG)_6$	F: TTACCAAAACGCCTCTCCAC R: GTTTCTTCCTTACGCCCCTGAAAT	60	170–190	8
CA421F	CF810704	$(CT)_{25}$	F: TCAAATTCAAAGCTCAAAATCAA R: GTTTAAGGATGATCCCGAAGCTCT	60	180–250	14
CA483F	CF810754	$(TC)_8$	F: GTCTTCCTCAGGTTCGGTTG R: GAACGGCTCCGAAGACAG	62	300–370	9
CA642F*	CF810880	$(CT)_{17}$	F: TGCCAACTCTGTTTAGGATGC R: GTTTATGCTGGAGCCAAAGAGAAA	60	350–390	23
CA787F	CF810934	$(GAA)_7$	F: TCCTCGTTCTCTCCCTCTCA R: GTTTCGCTGAAGTTGGAGTCCTT	60	270–300	5
CA794F	CF810941	$(GA)_{12}$	F: CGGTTGTCCCACTTCATCTT R: GTTTGAATTTGGCTTCGGATTC	60	220–290	10
CA855F	CF811000	$(GA)_{14}(CGA)_5$	F: CGCGTGAAAAACGACCTAAT R: GTTTACTCGATCCCTCCACCTG	64	250–300	10
CA94F	CF811011	$(AG)_7$	F: CACCCATTTCACGGAATCTC R: GTTTACTTGGTCGGGTGTTGTCTC	60	390–420	7
NA41	CF811380	$(CT)_{10}(CT)_7$	F: TTCCTTTAGTCGCGTCATCA R: GTTTAAGGTCGCTACGAGACTCCA	62	200–275	11
NA398	CF811369	$(AAAT)_5$	F: TCCTTGCTCCAGTCCTATGC R: GTTTCCTTCCACTCCAAGATGC	56	210–240	5
NA741	CF811540	$(TC)_9$	F: GCCGTCGCCTAGTTGTTG R: GTTTGATTTTGGGGGTTAAGTTTGC	58	240–290	14
NA800*	CF811589	$(TC)_{13}$	F: CAATCCATTCCAAGCATGTG R: GTTTCCCTAGACCAGTGCCACTTA	60	230–290	31
NA824*	CF811613	$(AG)_{17}$	F: AAATCGTTGGTTTGGCTCTG R: GTTTGGGCCGAAAAGAAATCGTAT	60	150–220	8
NA961	CF811674	$(TAC)_5$	F: TCAGACATGATTGGGGAGGT R: GTTTGGAATAATAGAGGCGGTGGA	60	205–220	6
NA1040	CF811165	$(TC)_{11}$	F: GCAACTCCCAGACTTTCTCC R: GTTTAGTCAGCAGGGTGCACAA	60	180–270	15
VCC_B3	AY842445	$(AG)_9$	F: CCTTCGATCTTGTTCCTTGC R: GTTTGATGCAATTGAGGTGGAGA	62	250–275	ND
VCC_H9	AY762677	$(CT)_{13}$	F: TCCGAGCCATTTAGTGTCAA R: GTTTACAAAAACCAAAAGCCATGC	62	250–275	13
VCC_I2	AY762678	$(CT)_{14}$	F: AGGCGTTTTTGAGGCTAACA R: TAAAAGTTCGGCTCGTTTGC	62	200–275	10
VCC_I8	AY762679	$(TG)_8$	F: TTCAGCATTCAATCCATCCA R: GTTTCTCTTCTCCAATCTCTTTTCCA	62	120–140	4
VCC_J1*	AY762680	$(CA)_6$	F: CTCATGGGTTCCCATAGACAA R: TGCAGTGAGGCAAAAGATTG	64	225–275	22
VCC_J3	AY762681	$(AAG)_{15}$	F: TGATTACATTGCCAGGGTCA R: TGGAAACAACCGGGTTACAT	58	150–200	8
VCC_J5	AY762682	$(TC)_{17}$	F: CCCCAACGGTCTTGATCTTA R: GTTTCCTCTCTCTCCAACCCCAGT	54	250–300	12
VCC_J9	AY762683	$(TG)_9(GA)_{23}$	F: GCGAAGAACTTCCGTCAAAA R: GTGAGGGCACAAAGCTCTC	62	100–200	10
VCC_K4	AY762684	$(TC)_{16}(TC)_{12}$	F: CCTCCACCCCACTTTCATTA R: GCACACAGGTCCAGTTTTTG	62	150–300	14
VCC_S10*	AY762685	$(CT)_{22}$	F: ATTTGGTGTGAAACCCCTGA R: GTTTGCGGCTATATCCGTGTTTGT	60	200–300	29

*amplifies multiple loci as determined by capillary electrophoresis.

Table 2 Cross amplification (A) and polymorphism (P) of EST-SSRs in 12 *Vaccinium* species. Two accessions per species were evaluated except in *Vaccinium oldhamii*, section *Ciliata*, where only one individual was available and polymorphism within species was + if two alleles were clearly visible

	A/P for *Vaccinium* species*											
Locus	*ov*	*br*	*vi*	*pa*	*cr*	*ma*	*da*	*te*	*el*	*co*	*ar*	*ol*
CA23F	+/–	+/–	+/–	+/+	+/–	+/–	+/+	+/+	+/–	+/+	+/–	+/–
CA112F	+/+	+/–	+/–	+/–	+/+	+/+	+/–	+/+	+/+	+/+	+/–	+/–
CA169F	+/–	+/–	+/+	+/–	+/+	+/+	+/+	+/–	+/+	+/+	–/–	–/–
CA236F	–/–	–/–	+/+	+/+	–/–	–/–	+/+	+/+	+/+	–/–	–/–	–/–
CA344F	+/–	+/–	+/–	+/–	+/–	–/–	–/–	+/+	+/+	+/+	+/–	+/–
CA421F	+/+	+/+	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/–
CA483F	+/–	+/+	+/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/–
CA642F	–/–	+/+	–/–	–/–	–/–	–/–	+/+	+/+	+/+	+/+	–/–	+/–
CA787F	+/–	+/–	+/–	+/+	–/–	–/–	–/–	–/–	–/–	+/+	+/–	+/–
CA794F	+/+	+/+	+/–	+/–	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
CA855F	+/–	+/–	+/–	+/–	+/+	+/–	+/+	+/+	+/+	+/+	+/+	+/–
NA398F	+/+	+/+	+/–	+/–	–/–	–/–	+/–	+/+	+/+	+/+	+/+	+/+
NA741F	+/–	+/–	+/+	+/+	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/–
NA800F	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
NA824F	+/–	–/–	+/–	+/–	–/–	+/–	–/–	+/+	+/+	+/+	–/–	+/–
NA961F	–/–	+/–	+/+	+/–	+/–	–/–	+/–	+/+	+/+	+/–	+/–	+/–
NA1040F	–/–	+/+	+/+	+/–	–/–	+/+	+/+	+/+	+/+	+/+	+/+	+/–
VCC_H9	–/–	–/–	–/–	–/–	–/–	–/–	+/–	+/–	–/–	+/–	–/–	–/–
VCC_I2	+/+	+/+	+/+	+/+	+/–	–/–	+/–	+/+	+/+	+/+	+/+	–/–
VCC_I8	–/–	+/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
VCC_J3	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–
VCC_J5	+/+	–/–	+/+	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/–
VCC_J9	–/–	+/+	–/–	+/+	+/+	+/+	+/+	–/–	+/+	+/+	+/+	+/–
VCC_K4	+/+	+/+	+/+	–/–	–/–	–/–	+/+	+/+	+/+	+/+	+/+	+/–
VCC_S10	+/–	+/–	+/+	+/+	+/–	+/+	+/+	+/+	+/+	+/+	+/+	–/–

*Species abbreviations, botanical sections and germplasm accessions used: (*ov*) *Vaccinium ovatum*, §*Pyxothamnus*, PI 555160, PI 555165; (*br*) *Vaccinium bracteatum*, §*Bracteata*, PI 554728, PI 554730; (*vi*) *Vaccinium vitis-idaea*, §*Vitis-idaea*, PI 555369, PI 555370; (*pa*) *Vaccinium parvifolium*, §*Myrtillus*, PI 555217, PI 555219; (*cr*) *Vaccinium crassifolium*, §*Herpothamnus*, PI 554900, PI 618186; (*ma*) *Vaccinium macrocarpon*, §*Oxycoccos*, PI 554972, PI 554996; (*da*) *Vaccinium darrowii*, §*Cyanococcus* PI 554904, CVAC 1356; (*te*) *Vaccinium tenellum*, §*Cyanococcus*, PI 618189, PI 555333; (*el*) *Vaccinium elliottii*, §*Cyanococcus*, PI 554916, PI 554919; (*co*) *Vaccinium corymbosum*, §*Cyanococcus*, PI 554880, PI 554881; (*ar*) *Vaccinium arboreum*, §*Batodendron*, PI 613194, PI 618120; (*ol*) *Vaccinium oldhamii*, §*Ciliata*, PI 555125.

total of 17 EST-SSR markers (11 CA and six NA) and eight VCC genomic SSRs were tested for cross-species amplification and polymorphism in 23 accessions from 12 species representing nine botanical sections of *Vaccinium* (Table 2). Two individuals per species were evaluated for polymorphism except in *Vaccinium oldhamii* where only one individual was available and it was scored as polymorphic within species if two alleles were clearly visible. PCRs were performed in a 10 μL volume containing 1× reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM of each primer, 0.25 units of Biolase *Taq* DNA polymerase (Bioline Inc.) and 2.5 ng genomic DNA. The optimum annealing temperature for a primer pair was determined by gradient PCR from 55 °C to 65 °C on an equimolar mix of the DNA templates used for screening. After initial denaturation at 94 °C for 3 min, DNA was amplified for 35 cycles in an Eppendorf Gradient thermocycler or an MJ Research Tetrad thermocycler programmed for a 40 s denaturation step at 94 °C, a 40 s annealing step at the optimum annealing temperature of the primer pair and a 40 s extension step at 72 °C. A final extension step was included at 72 °C for 30 min. For determination of allele numbers in the panel of 12 *V. corymbosum* accessions, fluorescently labelled forward primers were used and PCR products were sized on either an ABI 3100 sequencer (Central Services Laboratory, Oregon State University, Corvallis, OR) or a Beckman CEQ 8000 genetic analyser (Beckman-Coulter). For cross-species amplification, PCR products were separated on a 2% agarose gel and visualized after ethidium bromide staining.

Out of 1305 EST sequences examined, 977 (or 75%) unique sequences were found. A higher proportion of the sequences in the SSR-enriched genomic library were unique: 124 out of 136 sequences examined, which corresponds to 91% of the total. The EST library contained 114 unique

sequences with five or more microsatellite repeats (9% of all EST sequences). In comparison, the genomic library contained 39 unique sequences with five or more microsatellite repeats (29% of all enriched genomic sequences). Primer pairs were designed for 65 EST sequences (5% of all EST sequences) and 29 enriched genomic sequences (21% of all enriched genomic sequences).

From the EST libraries, 20 robust and polymorphic primers were obtained from 1305 sequences (an overall efficiency of 1.5%) (Table 1). From the enriched genomic library, 10 robust and polymorphic primers were obtained from 136 sequences (an overall efficiency of 7%) (Table 1). As expected, the EST library contained a higher percentage of trinucleotide repeats than the genomic library (21% vs. 8%). In both the EST libraries and the SSR-enriched genomic library, the AG/CT repeat motif was the most common (61% and 69% all repeat motifs, respectively). The AC/GT repeat motif was less common in the EST libraries than in the enriched genomic library (5% and 30% of all repeat motifs, respectively). Polymorphism was high and the allele number of 24 single locus SSRs in 12 blueberry accessions ranged from two to 15 per locus, and was 8.16 on average (Table 1). Five loci appeared to amplify multiple loci based on amplification of more than four fragments detected after separation by capillary electrophoresis. These multilocus SSRs include NA800, NA824, CA642F, VCC_J1 and VCC_S10 and the allele number ranged from eight to 31 (Table 1).

Cross-species amplification in a panel of 12 species representing nine sections ranged from 30% to 100%, and was 83% on average (Table 2). As expected, SSR loci derived from tetraploid *V. corymbosum* were most easily transportable to other members of the same §*Cyanococcus*. The SSRs failed to amplify more frequently in §*Oxycoccos*, §*Herpothamnus*, §*Batodendron* and §*Ciliata*.

We expect that these SSR markers will be useful not only for cultivar identification and diversity assessments in the National Clonal Germplasm Repository (NCGR) blueberry collection but also for ecological studies and linkage mapping, particularly in *Vaccinium* section *Cyanococcus*.

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